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Table of Contents

Cover.....1

SF 298.....2

A. Introduction..... 4

B. Body.....4

C. Key Research Accomplishments.....10

D. Reportable Outcomes.....11

E. Conclusions.....12

F. References.....12

G. Appendices.....13

H. Personnel who worked on the Project.....13

A. INTRODUCTION: Flow cytometry has become an important technique for rapid monitoring of receptor expression and proliferation in hematological tumors. In contrast it has been of limited use for the analysis of human solid tumors. Except for determination of DNA content and proliferation, monitoring of other cellular parameters by flow cytometry in breast tumors has been difficult. Most of these difficulties are posed by architectural characteristics of breast tumors where the tumor cells are embedded in stromal components containing extra-cellular elements such as collagen. As it is comparatively easy to isolate nuclei from human breast tumors, we have worked on the hypothesis that flow cytometry can be used for monitoring expression of nuclear markers of clinical significance. This could include nuclear hormone receptors (e.g., estrogen, progesterone, vitamin D) in combination with measurement of nuclear volume and DNA content. As the house keeping oncogenes, p53 and the proliferation marker, Ki-67 have important prognostic significance and are also expressed in nuclei, our objective was to ultimately develop multiparametric methods for rapid analysis of nuclear markers by flow cytometry.

Work accomplished to date has focused on: 1. Monitoring of nuclear hormone receptor expression in fresh/frozen and formalin-fixed/paraffin embedded breast tumors by flow cytometry and 2. Monitoring of nuclear volume, protein and DNA content of fresh and formalin fixed archival breast tumors and ductal carcinoma in situ. and 3. Development of a procedure for high resolution DNA flow cytometry of nuclei stained with DAPI. We believe flow cytometric evaluation of nuclear volume and marker expression can be used to refine histopathological grading of intermediate grade ductal carcinoma in situ.

The flow cytometric methods we have developed are rapid, highly sensitive and can determine not only the percentage of receptor positive nuclei in a heterogeneous breast tumor population but also measure antigen density of the individual nuclei and subpopulations. By multiparametric analysis, we have correlated expression of nuclear markers with DNA aneuploidy. We believe flow cytometric analysis of nuclear hormone receptors and nuclear volume in combination with rapid and high-resolution analysis of DNA content can be an important quantitative multiparametric method for diagnostic and prognostic studies in breast cancer.

B. BODY: The main specific aims of this project were to: 1. Collect breast tumor biopsies and archival tissue blocks from local and outside repositories. 2. Develop preparatory methods for flow cytometric analysis of nuclei obtained by enzymatic digestion of archival breast tumors 3. Study the resulting single nuclei for marker expression (estrogen, androgen and vitamin-D hormone receptors), DNA content (aneuploidy and S-phase fraction) and nuclear volume.

B.1. Breast Tumor Collection was based on samples collected from the Jackson Memorial Medical Center, Miami, Suburban Hospital, Bethesda, MD, and NIH sponsored CHTN tissue network in Birmingham, AL. Most of the samples from our local patients represent a broad spectrum of racial and ethnic populations unique to South Florida. From these resources, frozen and formalin fixed/paraffin embedded blocks of breast tumors representing a variety of histopathological grades and types of tumors were collected. In addition, we collected formalin fixed/paraffin embedded female breast tumors from India and Egypt. Breast tumors from male patients were collected from Jamaica, West Indies. Our interest in breast tumors from India, Egypt and Jamaica was due to the reported early onset and aggressive behavior of breast tumors in these special populations.

B.2. Nuclear Estrogen Receptor Expression and DNA ploidy: This study was based on

examination of more than 100 breast tumor samples of different histological grades and types collected from USA and Egypt. Thick (50 micron) and thin (5 micron) sections were cut from paraffin blocks of formalin fixed breast tumors for analysis by laser flow cytometry and immunohistochemistry, respectively. Thick sections were enzymatically digested for release of nuclei, processed for antigen retrieval and studied for receptor expression and DNA content (aneuploidy and cell cycle distribution) by flow cytometry using a Coulter XL flow cytometer with argon laser excitation. Thin sections cut were stained by immunohistochemistry and studied for ER expression and histopathological grading.

Data from immunohistochemistry and flow cytometry was analyzed for percentage of ER positive cells and DNA content of the subpopulations. Flow cytometric data was compared with immunohistochemical determinations on ER positive or negative status of the tumors examined under a microscope. Correlation between ER expression by flow cytometry and the immunopathological evaluation of the tumors was undertaken. Drs. Jinghai Wen and A. Khayat of the National Cancer Institute, Cairo who were the primary investigators on this project, are preparing data from this study for publication.

B.3. Androgen, Vitamin-D Receptor Expression and DNA content of Human Breast Tumors: In the second year of the project, Dr. Rao, a UICC/ICRETT International scholar, and Dr. Poonam Arya, Research Associate, used our flow cytometric methods to monitor expression of androgen and vitamin-D nuclear hormone receptors in breast tumors. Dr. Rao focused on breast tumors collected in Manipal, India while Dr. Arya worked on breast tumor samples from the USA. We had chosen the Indian and the Jamaican breast tumors for study due to reported early onset and aggressive course of breast tumors in these populations. Subsequently we added a cohort of male breast tumors from Jamaica to the study. Methods for both antigen retrieval and staining had to be modified for analysis of tumors obtained from outside USA as the fixation and embedding methods used in these labs differed from those used in our labs.

Thick sections were enzymatically digested for isolation of nuclei, which were then processed for antigen retrieval and stained with the anti-AR or vitamin-D primary antibodies and labeled secondary antibodies. Thin sections were stained for immunohistochemistry and studied for AR/VDR expression and histopathological grading. Gated analysis was used to compare the expression of these receptors in sub-populations with diploid and aneuploid DNA content.

Out of the 25 **female breast tumors** analyzed, 13 tumors had diploid DNA content, while 12 had distinct aneuploid sub-populations with triploid or hypo-tetraploid DNA content. The percentage of AR positive nuclei decreased with increase in tumor grade. Aneuploid subpopulations had higher percentage of AR positive nuclei as compared to diploid sub-populations.

Besides determining the percent of AR positive nuclei in a sub-population, we compared the ratio of the mean log fluorescence channel (MFC) value of the isotype and the antibody treated samples as a measure of antigen density. Variable MFC ratios were obtained. For example, in diploid tumors the MFC ratio varied from 1.87 to 6.5. In tumors with triploid sub-populations and the near-tetraploid tumors, the MFC ratio ranged between 2.28 to 6.80 and 2.3 to 6.79, respectively.

Out of 33 **male breast tumors** analyzed, 25 tumors were predominantly diploid and the remaining 8 tumors contained distinct aneuploid populations. In diploid tumors, the percent of AR positive nuclei varied from 5 to 61 with a mean of 31 ± 14 . In triploid sub-populations, the range was from 23 to 64 with a mean of 44 ± 15 and in subpopulations with greater than 4N DNA content; the range was from 16 to 61

with a mean of 40 ± 16 . AR expression in male breast tumors was in general lower than that of the female breast tumors.

The percentage of **vitamin D receptor** positive nuclei varied from 28 to 86 with a mean of 66 ± 21 in diploid nuclei. In triploid tumors, the range was 68 to 91 with a mean of 70 ± 16 . In sub-populations with greater than 4N DNA content the range was from 36 to 91 with mean of 63 ± 20 . VDR expression in breast tumors was highly variable and diploid sub-populations had higher VDR expression than aneuploid sub-populations. Data from this study was recently published in Clinical Cytometry (1) and a reprint is attached (Appendix 1).

Dr. Rao, used the flow cytometric methods to compare and correlate the expression of AR and vitamin-D in paraffin embedded breast tumors from Indian Patients with other clinical parameters. Paraffin embedded blocks from 76 primary infiltrating ductal (ID) breast carcinoma and benign breast tumors from the Department of Pathology, Kasturba Medical College, India were used for this study. Approximately 44% of the ID tumors were diploid while the remaining tumors had a sub-population of nuclei with distinct triploid (36%) or tetraploid (10%) DNA content. Percent AR positive nuclei ranged from 16-66% in the ID tumors and 36-67% in benign tumors. The percent of vitamin-D receptor positive nuclei was 14-89% and 2-75% in ID tumors and benign tumors, respectively. Benign tumors had significantly higher AR and significantly lower Vitamin-D expression than nuclei from the malignant tumors. There was a strong correlation between the percent of receptor positive nuclei and antigen density.

The pooled data from patients of pre-and post-menopausal group with an average age of 47 ± 13.2 yrs (28 to 80 years) revealed percentage of vitamin-D positive nuclei decreased with the patients' age. However, a similar co-relation between patients' age and AR expression was not seen. Interestingly, both AR and vitamin-D expression decreased with an increase in patients age in the pre-menopausal group, and AR expression increased with the increase in patients age in post-menopausal group. The AR-vitamin-D expression and tumor grade in the pooled data revealed weak correlation. In the pre-menopausal women, a weak correlation between vitamin-D expression and clinical stage was observed. The percentage of vitamin-D positive nuclei decreased and AR positive nuclei increased along with the tumor grade. Whereas, both the percent AR/vitamin-D positive nuclei decreased among the women in post-menopausal group with advanced stage of malignancy. No correlation was seen between DNA ploidy and receptor expression. The percentage of AR/ vitamin-D positive nuclei decreased in both pre and post-menopausal group with the increase in tumor size. No statistically significant correlation was observed between other clinical parameters such as tumor grade, nodal status, and metastasis and AR/Vitamin-D expression. A manuscript based on this data is being revised for publication (2).

B.4. Nuclear Volume versus DNA content of nuclei isolated from Human breast tumors.

We have described development and use of a high-resolution flow cytometer, which can simultaneously measure nuclear volume and DNA content of tumor nuclei (3-5). This instrument by using multiparametric analysis of electronic nuclear volume versus DNA content can differentiate between normal and tumors cells and identify tumor cells in secondary sites such as lymph nodes or bone marrow of breast tumor patients (5). Our first effort was to develop staining protocols for optimizing multiparametric analysis of breast tumor cells in this cytometer. We explored different combinations of DNA binding dye concentrations, pH and tonicity to identify conditions, which provide the best possible data for multiparametric and simultaneous evaluation of nuclear volume and DNA content of nuclei prepared from a tumor biopsy (4). However, when we tried to use these methods on a larger set of fresh

tumors, we got variable results. In a subsequent study, we explored a variety of methods for processing formalin-fixed/paraffin embedded tissues for determination of nuclear volume and DNA content. These observations were recently published (reprint Appendix # 2) and as seen in Figure 1, we developed procedures for obtaining high-resolution data on nuclear volume and DNA content of formalin fixed/paraffin embedded tissues and tumors.

During this study, we observed that DAPI as a stain for DNA had some unusual characteristics. In the following section, we have described our observations on the staining characteristics of DAPI, which may be used to differentiate between a normal and tumor nuclei and sub-populations of nuclei with S-phase DNA content.

B.5. Metachromasia of Normal vs. Breast Tumor Nuclei: DAPI, a DNA binding fluorochrome has been extensively used for flow cytometric determination of nuclear DNA content and cell cycle distribution. In our studies on nuclear volume vs. DNA content of human tumors (5,6), we routinely used either the commercially available NIM-DAPI (presumably containing 10 $\mu\text{g/ml}$ of DAPI) obtained from NPE Systems, Inc, Pembroke Pines, Fl.) or our DAPI formulation containing 3 $\mu\text{g/ml}$ of DAPI in phosphate buffered saline, pH 7.0, with 1% NP-40 (4). In both of these procedures, nuclei were isolated by lysis of the cells either by a hypotonic solution, detergent (e.g. NP-40) or a combination of the two. In most cases this results in nuclear preparations, which can be analyzed after a few minutes of staining and can be stored for up to 24 hrs in a refrigerator. In most of the human breast tumors stained by our DAPI formulation, the CV of the G_0/G_1 peaks ranged between 2-4. During the course of these studies, we were intrigued by some of the tumor samples which failed to yield high resolution DNA histograms after staining with NIM-DAPI while aliquots of the same tumor stained in parallel with propidium iodide/hypotonic citrate (7) gave reasonably good histograms. Most of the "difficult specimens" had broad CV's and in dot plots of volume vs. DNA, the distribution was skewed to the right.

As our earlier studies and observations by other workers (4) had suggested that DAPI concentration may affect CV of the DNA histograms, studies were carried out to see if dilution or washing of the breast tumor specimens with PBS would affect CV of the DNA histograms. Figure 2A shows DNA histogram of nuclei from a breast tumor and TRBC stained with NIM-DAPI. The TRBC were recorded at channel 50 and the tumor nuclei had broad CV and indicate the presence of two sub-populations. Dot plot of DNA vs. nuclear volume (Fig. 2D), show a prominent slope, which accounts for the broad CV of the DNA distribution. Histogram 2B and dot plot 2E are of the sample shown in Fig 2A after dilution with 1:3 parts of PBS. The slope of the DNA vs. nuclear volume (Fig. 2E) is reduced in this sample. Figure 2C and 2F are of the nuclei after they were centrifuged and resuspended in saline. The CV of this distribution has significantly improved and the slope seen in Fig. 2D was not evident in the DNA vs. nuclear volume plots (2F). These observations would suggest that the broad CV and the slope of the DNA vs. volume plots was due to the presence of excess dye which was removed by dilution and/or washing of the nuclei.

To further explore the spectral basis for the broad CV observed, we used a Quanta NPE analyzer in which the photodiode has been replaced with filters, beam splitters and photo-multiplier tubes for collection of blue (450nm) and red (580nm) fluorescence simultaneously with electronic nuclear volume.

Dot plots in Figure 3 show electronic volume (Y-axis) vs. blue fluorescence (X-axis) emission of nuclei stained with NIM-DAPI (A), 1:2 dilution of NIM-DAPI (B) and our DAPI solution (C), respectively. This tumor had three major populations of nuclei with 2C, hypo-4C and 4C DNA content. The CV of the 2C peak (blue emission) was 4.97, 3.10 and 2.02, in Figures 3 A to C, respectively. Dot plots 3D-F, record electronic volume vs. red fluorescence emission of the nuclei stained with the three DAPI formulations. It is clear that in nuclei stained with NIM-DAPI (Fig.3D), there was broad emission of red fluorescence, not seen in nuclei stained with the lower concentrations of NIM-DAPI or our DAPI (Fig. 3 E, F).

From these studies, we concluded that in contrast to the normal nuclei with small volume, tumor cell nuclei stained with higher concentrations of DAPI have a pronounced red emission which can be used to discriminate between normal and tumor cells in a heterogeneous population.

B.6. DAPI identifies Sub-populations of S-Phase Cells: By comparing blue vs. red emission of DAPI stained nuclei, we can identify two distinct sub-populations of nuclei with S-phase DNA content.. This phenomenon is shown in Fig 4 where, nuclei with 2C and 4C DNA content (corresponding to G_0/G_1 and G_2 DNA content) had proportional amounts of red and blue emission, while the sub-population with S-phase DNA content had two distinct sub-populations which differ in their red fluorescence emission (arrow). In preliminary experiments, we determined that this phenomenon was not related to dye/cell concentration or to the presence of RNA, as digestion with RNase did not abolish the distinction. A paper based on these observations is being revised for publication.

B.7. Correlation of Nuclear Volume and Grading of Breast Tumors: For histopathological grading of human breast ductal carcinoma in situ, nuclear size, chromatin texture and necrosis are considered to be three important parameters (6,7). At present most of the histopathological studies are carried out visually using a microscope and thus are slow and often based on examination of a small number of cells.

In the NASA/ACS flow cytometer, simultaneous measurement of electronic volume versus DNA content can identify sub-populations in a heterogeneous solid tumor and possibly discriminate between normal and tumor cells at metastatic sites (5). We have recently developed methods for rapid determination of nuclear size and DNA content of nuclei isolated from cryopreserved and formalin fixed-paraffin embedded human breast tumors (8). Our preliminary data showed that this method could be used to detect differences in nuclear volume and DNA content of tumor cells with a level of precision unattainable with standard flow cytometric analysis in conventional flow cytometers (Figure 1). Our working hypothesis is that measurement of nuclear volume versus DNA content of human ductal carcinoma in situ in combination with monitoring the expression of nuclear hormone receptors by our flow cytometric methods can be a useful tool for accurate grading of breast tumors.

We undertook a study on correlation of DNA content and nuclear volume in ductal carcinoma in situ. DNA histograms (Figure 5, A-D) and contour plots of DNA vs. Electronic nuclear volume (Figure 5,E-H) are of nuclei isolated from normal breast tissue (A, E), and from tumor biopsies of patients diagnosed with ductal carcinoma in situ (DCIS, B-D, F-H). Trout red blood cells were added to the samples as an internal standard and recorded at channel # 50 (Figure 5, A-C) or at # 29 (Figure 5, D).

In normal breast tissues, nuclei with diploid (2C) DNA content were recorded at channel # 82 with a corresponding small peak of cells with G_2/M DNA content at channel # 165. In contour plots, the normal diploid breast nuclei had electronic volume similar to that of the TRBC used as controls (Figure 5E).

Electronic Nuclear Volume vs. DNA content contour plots of the three representative DCIS samples shown in Figure 5 (F-H) show the variation in electronic volume of the nuclei with diploid, tetraploid and hexaploid DNA content. In contour plot 5F, the nuclei with both diploid and tetraploid DNA content were much larger than TRBC but had similar electronic volume. In contrast in contour plot 5G, while most of the nuclei with diploid DNA content had electronic volume similar to that of the TRBC, the nuclei with tetraploid DNA content were 2 to 3 times larger.

DNA histogram in Figure 5D shows TRBC at channel 29, a diploid peak at channel 52 and an aneuploid (hexaploid) peak at channel 150. Contour plot 5H shows that in contrast to the nuclear volume of the tetraploid nuclei in Figure 5G, the hexaploid nuclei in this tumor had relatively smaller volume.

B.8. Identification of Sub-populations Based on Nuclear Volume vs. DNA Content:

Plotting of electronic nuclear volume vs. DNA content in contour plots can reveal the presence of sub-populations which may not be readily recognizable in DNA histograms or in light scatter vs. DNA plots. As shown in DNA histogram 6A, a distinct hyper-diploid population with DNA index of 1.15 is recognizable as nuclei with large electronic volume in the nuclear volume vs. DNA contour plots shown in Figure 6B. In DNA histogram 6C, the twin peaks of nuclei with tetraploid DNA content (DI, 1.79, 1.98) are resolved as two distinct populations with similar electronic nuclear volume in the contour plot 6D.

In DNA histogram 6E, three main peaks of diploid, hypo-tetraploid (DI 1.73) and tetraploid (DI 2.03) DNA content are seen. The ENV vs. DNA plot of this tumor in Fig 6F show that both the diploid and the hypo-tetraploid populations had sub-populations that differ in their nuclear volume. On the basis of electronic nuclear volume vs. DNA contour plots one can recognize at least eight sub-populations in this DCIS sample.

We have submitted grant applications to DOD and NIH (May- June, 2004 deadline) for continuation of studies on grading of DCIS on the basis of nuclear volume and DNA content. A manuscript based on analysis of nuclear volume versus DNA content is being prepared for submission for publication.

B.9. Correlation of Nuclear Hormone Receptor Expression, Nuclear Volume and other Marker Expression in Fresh and Formalin Fixed/Paraffin Embedded Tumors: In the final phase of the project, we have focused on multiparametric flow analysis for correlating expression of the nuclear markers with DNA content and nuclear volume.

Nuclei isolated from fifty one-formalin fixed/paraffin embedded tumors, and twenty frozen breast tumors and 15 normal breast samples were stained with DAPI or PI for DNA and FITC for protein content. Data correlating nuclear volume, protein and DNA content with tumor grade is being analyzed now.

The prototype NASA/ACS flow cytometer in our lab. had a single channel solid-state detector for monitoring of fluorescence of nuclei stained with DAPI. This detection system although good enough for DNA analysis, was not adequate for monitoring fluorescence of hormone receptor and other markers such as p53 and Ki-67. Our instrument was modified by the addition of two highly sensitive photo multiplier tubes and optical mirrors and filters for the detection of low-level two-color fluorescence. The hydraulics

was improved by addition of an automatic sample introduction system and other refinements in hardware and software.

After testing of these additional refinements, we ran experiments to see if labeled antibody fluorescence can be used in multiparametric setting of this instrument with nuclear volume and DNA content. Nuclei isolated from formalin-fixed/paraffin-embedded and fresh (frozen) breast tumors were stained with phycoerythrin labeled anti-androgen and progesterone antibodies. We can now measure expression of nuclear hormone receptors in archival (Figure 7) and fresh/frozen breast tissues and tumors (Figure 8) in the flow cytometer with capability for simultaneous monitoring of nuclear volume and DNA content.

During use of the modified instrument for dual receptor expression work, it became evident that we could not use it for simultaneously monitoring and correlating expression of two different fluorochromes with DNA content and volume. Due to the large differences in emission intensity of DAPI and other fluorochromes, a whole new optical filter set with software based color compensation was required to carry out the proposed studies. NPE systems System Inc. had to develop the new software, which was installed in our laboratory in June 2004. With the availability of the compensation software, we are now ready to finish our studies on multicolor analysis of marker expression and correlate it with DNA content and nuclear volume. We have started these studies and we expect to examine 50 stored tumors and normal tissue within the next month to conclude the project by end of July 2004.

In the subsequent few months, we plan to prepare three manuscripts on: 1. Nuclear volume and DNA content of breast tumors, 2. Nuclear marker expression and its correlation with nuclear volume in breast tumors and 3. Metachromatic studies on nuclei stained with DAPI.

C. KEY RESEARCH ACCOMPLISHMENTS:

- Analyzed breast tumors of different grades and types for the expression of nuclear estrogen receptor expression. Flow cytometric data is being compared and correlated with immunohistochemical data.
- Studied androgen and vitamin-D expression in female and male breast tumors collected from South Florida, India, Egypt and Jamaica. These observations were recently published in the Clinical Cytometry journal.
- Studied nuclear androgen and vitamin-D expression in Indian breast tumors and correlated expression with clinical parameters of age, menopause, tumor grade, tumor type and co-expression of AR and VDR receptors. A manuscript based on these observations is being revised for publication.
- Developed methods for simultaneous analysis of nuclear volume and DNA content of ductal carcinoma in situ by a high-resolution flow cytometer. Completed study of more than 100 ductal carcinoma in situ for correlation of nuclear volume vs. DNA content with histopathological grading of the tumors. Data is being analyzed for publication.
- Developed staining protocols for simultaneous staining of breast tumor nuclei with antibodies for nuclear receptors and correlation of receptor expression with nuclear volume. This part of the stated aim was delayed due to upgrading of instrumentation for performance of two-color analysis.
- Obtained and Installed software for performing compensation for analysis of nuclei stained with two fluorochromes. This modification should allow us to finish the final phase of the project by the end of July 2004.

D. REPORTABLE OUTCOMES:

- Flow cytometric protocols have been developed for monitoring of nuclear hormone receptor expression in archival formalin fixed/paraffin embedded breast tumors.
- Androgen expression is reduced in high-grade tumors as compared to low-grade tumors.
- In multiploid female and male breast tumors, aneuploid sub-populations have higher percent of AR positive nuclei than diploid populations.
- Male breast tumors have relatively lower AR expression than corresponding female breast tumors.
- In female breast tumors, vitamin D receptor expression was higher in diploid than in aneuploid nuclear sub-populations.
- In multiploid female breast tumors, the aneuploid sub-populations did not have significantly greater percentage of vitamin-D positive nuclei than the diploid sub-populations in the same tumor.
- Nuclear volume versus DNA content may be a useful parameter to identify sub-sets of human breast ductal carcinoma in situ. Correlation of nuclear volume with tumor grade may allow us to refine the grading of DCIS.
- Two-color analysis (red vs. blue emission) of nuclei stained with DAPI may allow discrimination of normal vs. tumor cells as well as identify sub-populations in s-phase.

D.1. Presentations at Meetings:

1. A.Krishan P. Arya, I. Andritsch, SK Adiga, and M. Nadji. Flow cytometric analysis of androgen and vitamin D receptor expression in formalin fixed/ paraffin embedded archival prostate and breast tumors. Proc. Amer. Assoc. Cancer Res. Annual meeting. March 2002. Abstract # 4555.
2. A. Krishan, P. Arya, I. Andritsch and M. Nadji: Flow cytometric analysis of androgen receptor expression in archival human breast tumors. ERA of Hope DOD meeting. September 25-28, 2002. Poster Presentation.
3. A. Krishan, P. Arya, M. Nadji and I. Andritsch. Androgen and vitamin D receptor expression in human breast tumors. Proc. Inter. Soc. Anat. Cyto. San Diego, 2002.
4. A.Krishan, R. Hamelik and C. Milikowski. Flow cytometric monitoring of nuclear volume and DNA content of cells from body fluids of cancer patients. Proc. Amer. Assoc. Cancer Res. Annual meeting. March 2003. Abstract # 2501.
5. A.Krishan, P. Arya, P. Ganjei-Azar, S.E. Shirley, C.T.Escoffery, M. Nadji. Androgen and vitamin D receptor expression in archival human male and female breast tumors. Proc. Amer. Assoc. for Cancer Res. 2004 meeting. Poster presentation.
6. A. Krishan, R. Cabana, R. Hamelik, P. Ganjei- Azar. Flow cytometric analysis of electronic nuclear volume and DNA content of cells in human body fluids. Proc. Proc. Inter. Soc. Anat. Cyto. Montpellier, France, 2004. Slide presentation from platform.

D.2. Papers Published:

Krishan A, Cabana R. Flow Cytometric Analysis of Electronic Nuclear Volume and DNA Content in Normal Mouse Tissues. *Cell Cycle*. 3:380-383. 2004.

Krishan, A., P. Arya, P. Ganjei-Azar, S. E. Shirley, C. T. Escoffery, and M. Nadji. Androgen and Vitamin D Receptor Expression in Archival Human Breast Tumors. *Clinical Cytometry*. 58B: 53-60. 2004.

E. CONCLUSIONS:

The work proposed in this project was heavily dependent on a new instrument of which only a prototype existed in our laboratory at the time this grant was funded. Subsequently this instrument was upgraded to perform two-color fluorescence work, which meant design of new optical bench while maintaining the unique feature of measuring volume. This was accomplished in fall of 2002. We subsequently found that due to the large differences in fluorescence emission of DAPI and other fluorochromes, a mechanism for compensation of the emission spectra was needed. The NPE Systems Inc. recently developed this software package and installed it on our instrument in June 2004. Work done in our laboratory has been instrumental in developing this instrument, which will be available to the wider scientific community as the NPE Quanta analyzer sold by Coulter Beckman. The PI and his associates in this project have no monetary or commercial association with NPE systems Inc, which is going to manufacture this unit to be sold by Coulter Beckman.

One of the problems we did not anticipate was related to the availability of fresh breast tumor tissues. Although getting archival paraffin embedded tumors was relatively easy, obtaining fresh breast tumor tissue locally was very difficult. This was probably due to the early detection of small lesions and tumors by mammography and the priorities of using the small biopsies or fine needle aspirates for diagnosis. In most cases only fine needle aspirates were available which would not provide enough material for our studies. We filled an application with the NIH sponsored CHTN network and since 2003, they have provided us with fresh/frozen breast tumor and normal breast samples. However, the collection and shipping process is relatively slow and only now (May, 2004) we have collected enough samples of normal and breast tumors for the two-color work.

Methods developed thru this DOD grant made it possible to simultaneously monitor nuclear volume, DNA content and receptor expression of human breast tumors fixed in formalin and embedded in paraffin. We believe these methods are highly quantitative, reliable and can be used for refining the stratification and grading of breast tumors. Based on data generated in this project, we have submitted two grant applications to DAMD/DOD (for the May 11, 2004 deadline) and NIH (revised application submitted for July 1, 2004 deadline) for use of these technologies for correlating marker expression with clinical outcome in patients who remain disease free versus patients who develop invasive breast tumors.

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G. APPENDICES:

I. Reprint: Krishan, A., P. Arya, P. Ganjei-Azar, S. E. Shirley, C. T. Escoffery, and M. Nadji. Androgen and Vitamin D Receptor Expression in Archival Human Breast Tumors. Clinical Cytometry. 58B: 53-60. 2004.

II. Reprint: Krishan A, Cabana R. Flow Cytometric Analysis of Electronic Nuclear Volume and DNA Content in Normal Mouse Tissues. Cell Cycle. Cell Cycle. 3:380-383. 2004.

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Androgen and Vitamin D Receptor Expression in Archival Human Breast Tumors

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Background: The present study was undertaken for quantitation of androgen (AR) and vitamin D (VDR) receptor expression in human male and female breast tumors by flow cytometry.

Methods: Nuclei isolated from sections of paraffin-embedded tumors by pepsin digestion were treated for antigen unmasking and incubated with antibodies to AR and VDR. Flow cytometric analysis was used to determine the percentage of receptor-positive nuclei with fluorescence greater than 95% of the isotype nuclei. Mean log fluorescence channel values were used for comparing antigen density of the isotype and the antibody-treated nuclei.

Results: Six of 23 female breast tumors had aneuploid DNA content. Nineteen of 20 estrogen receptor-positive female tumors by immunohistochemical analysis (IHC) were also AR positive by flow analysis. Aneuploid subpopulations had higher percentages of AR-positive nuclei than did diploid populations. Eight of 33 male breast tumors had aneuploid DNA content. Twenty-three of 33 male breast tumors were AR positive by flow analysis compared with six that were AR positive by IHC. Six AR-positive (IHC) male tumors were also AR positive by flow analysis. VDR expression was higher in diploid female tumors than in aneuploid tumors.

Conclusions: Lack of a strong correlation between IHC and flow analysis may be due to differences in criteria used for identification of receptor-positive and -negative tumors by the two methods. © 2003 Wiley-Liss, Inc.

Key terms: androgen receptor; female breast tumor; flow cytometry; male breast tumor; vitamin D receptor

Breast cancer is one of the leading malignancies, and because breast tissue in general is hormone sensitive, hormonal therapy is used for prevention and treatment of this malignancy(1). Receptors for estrogen (ER), progesterone (PR), androgen (AR), and vitamin D (VDR) are present in normal human breast and tumor cells(2–7). The predictive and prognostic significance of ER and PR expression in breast cancer has been elucidated in several recent studies(7,8). In comparison, the significance of AR and VDR expression in human breast tumors is less clear. Several investigators have used cytosolic (dextran-charcoal), reverse transcription polymerase chain reaction, or immunohistochemical (IHC) assays to study AR expression in human breast tumors. Kuenen-Boumeester et al.(4) reported that 76% of breast tumors are AR positive, whereas 9% of AR positive tumors are negative for ER and PR expression. In 13% of tumors (all grade III), no detectable expression of ER, PR, or AR was seen. In a recent IHC study, Selim et al.(8) reported that 19 of 57 ductal carcinomas in situ were AR positive, and no association be-

tween AR and ER expression or between AR expression and degree of differentiation was seen.

Although the role of VDR in growth regulation and differentiation of breast tumor cell lines has been confirmed in several in vitro studies(9), conflicting reports have appeared on the prognostic value of VDR expression in human breast tumors(10–12). Freake et al.(6) found that, in human breast tumor cells, VDR expression is at concentrations higher than previously described, and Bu-

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ras et al.(13) reported that high VDR expression is seen in well-differentiated tumors as compared with less differentiated tumors.

Most of the earlier work on hormone receptor expression was based on ligand-binding assays, which measure cytosolic receptor expression. With the availability of receptor-specific antibodies, IHC has replaced ligand-binding assays for clinical evaluation of hormone receptor expression in human breast tumors(14,15). Because ligand-binding assays are based on determination of the receptor content in the cytosol, they cannot differentiate between receptor expressions of normal and tumor cells. The IHC methods are based on examination of a small number of cells under a microscope and are at best semi-quantitative.

Flow cytometry is used extensively for monitoring of receptor expression in human hematopoietic cells. Flow cytometry offers the advantage that quantitative data collected by rapid analysis of a large number of cells can be used for multiparametric correlative studies. However, use of flow cytometry for monitoring of hormone receptor expression in human solid tumors has been hampered by difficulty in obtaining single-cell suspensions. Because most of the antibodies used for IHC detection of the hormone receptors are specific for the nuclear receptors, we have used enzymatic procedures for isolating nuclei from archival paraffin-embedded tumors for flow cytometric determination of hormone receptor expression. In previous publications, we described methods for analyses of ER, PR, and AR expression(15-17) in human breast and prostate tumors. In the present study, we used these flow cytometric methods for monitoring AR and VDR expression in formalin-fixed, paraffin-embedded archival human male and female breast cancers.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded human breast tumor blocks (from 25 female and 35 male patients) were obtained from the Departments of Pathology at the University of Miami and the University of West Indies in Jamaica. Twenty-three of 25 female breast tumors and 33 of 35 male breast tumors were successfully processed for antigen retrieval and receptor expression. Two of 25 female and two of 35 male breast tumors did not provide adequate data for inclusion in this report. Tables 1 and 2 list the histopathologic and immunocytochemical characteristics of the tumors analyzed. A section stained with hematoxylin and eosin was used for histopathologic examination and selection of material for flow cytometric studies. Two 25- μ m sections from each block were deparaffinized, rehydrated in a descending ethanol series, and resuspended in 2 ml of phosphate buffered saline (PBS).

Enzyme Digestion and Antigen Retrieval

For antigen unmasking (antigen retrieval), sections were incubated in citrate buffer (0.01 M at pH 6.0) at 80°C for 2 h. After cooling at room temperature for 15 min, tissue sections were washed with PBS. For nuclear isolation, sections were digested in 3 ml of pepsin (0.05% in

Table 1
23 Female Breast Tumors for Which Comparative
Immunohistochemical Data on ER Expression Are Available*

Case no.	Type	DI	ER (IHC)	%AR ⁺ (FCM)
1	IDC	1.0	+	41
2	DCIS	1.0	+	56
3	LIS	1.0	+	49
4	LOB	1.0	+	47
5	IDC	1.0	+	79
6	IDC	1.6	+	50
7	IDC	1.0	+	77
8	IDC	1.0	+	63
9	DCIS	1.6	-	40
10	IDC	1.0	+	40
11	IDC	1.9	+	40
12	LIS	1.0	+	26
13	LIS	1.0	+	65
14	MED	1.0	+	71
15	IDC	1.6	+	37
16	IDC	1.0	+	45
17	IDC	1.0	+	37
18	IDC	1.7	+	71
19	IDC	1.8	+	44
20	TUB	1.0	+	9
21	LOB	1.0	+	39
22	IDC	1.0	-	35
23	IDC	1.0	-	42

*AR, androgen receptor; COM, comedo carcinoma; DCIS, ductal carcinoma in situ; DI, DNA index; ER, estrogen receptor; FCM, flow cytometry; IDC, infiltrating ductal carcinoma; IHC, immunohistochemical analysis; MED, medullary carcinoma; TUB, tubular carcinoma.

normal saline, pH 1.65; catalog no. P-7012, Sigma, St. Louis, MO) for 30 min at 40°C in a water bath. Tubes were periodically vortexed during the incubation. Examination of the digests under a microscope was used to confirm the isolation of nuclei. Proteolytic action was stopped by the addition of an equal amount of PBS containing 3% fetal bovine serum. The nuclear pellet retrieved by centrifugation for 16 min at 1,000g in a Beckman TJ-6 centrifuge was washed with PBS and resuspended in 1 ml of PBS.

AR Staining

For the isotype control, 250 μ l of the pepsin-digested sample was incubated with 200 μ l of the anti-androgen antibody (MU256-UC, clone F39.4.1, BioGenex, San Ramon, CA) diluted 1:45 in PBS for 18 h at 37°C. Mouse immunoglobulin (Ig) G1 κ (catalog no. M 5284, MOPC 21, Sigma) diluted 1:180 in PBS was used. All samples were washed with 3% fetal bovine serum in 0.05% Triton-X in PBS, centrifuged at 1,000g in a Beckman TJ-6 centrifuge for 16 min. The secondary goat anti-mouse antibody (fluorescein isothiocyanate [FITC] conjugated, catalog no. F-4143, Sigma) at a dilution of 1:80 was mixed with the nuclear suspension, and the tubes were incubated in dark for 45 min. After a wash with PBS containing 0.05% Triton-X and 3% fetal bovine serum, the tubes were centrifuged and the pellets were stained with propidium iodide (25 μ g/ml in PBS containing 1 mg/ml of RNase) at 37°C for 20 min.

Table 2
33 Male Breast Tumors Analyzed*

Case no.	Type	DI	ER (IHC)	AR (IHC)	%AR ⁺ (FCM)
1	IDC	1.0	+	—	43
2	IDC	1.0	+	—	30
3	IDC	1.0	+	—	28
4	MET	1.0	+	—	21
5	IDC	1.0	+	—	26
6	IDC	1.0	+	—	13
7	IDC	1.0	+	—	6
8	IDC	1.0	+	—	5
9	Apocrine	1.0	+	—	1
10	Angiosarc	1.0	—	—	43
11	IDC	1.0	+	—	52
12	IDC	1.0	+	—	52
13	MET	1.2	—	+	23
14	MET	1.0	—	+	36
15	Mucinous	1.0	+	—	11
16	IDC	1.0	+	—	30
17	IDC	1.0	+	—	28
18	IDC	1.6	+	+	23
19	IDC	1.0	+	—	23
20	IDC	1.6	+	+	25
21	IDC	1.0	+	—	19
22	IDC	1.6	+	—	41
23	IDC	1.5	+	—	28
24	IDC	1.6	+	+	29
25	IDC	1.6	+	+	28
26	MET	1.5	+	—	13
27	IDC	1.0	+	—	40
28	IDC	1.0	—	—	17
29	IDC	1.0	—	—	14
30	IDC	1.0	—	—	16
31	IDC	1.0	—	—	41
32	IDC	1.0	+	—	43
33	IDC	1.0	+	—	47

*AR, androgen receptor; DI, DNA index; ER, estrogen receptor; FCM, flow cytometry; IDC, infiltrating ductal carcinoma; IHC, immunohistochemical analysis; MET, metastatic carcinoma.

VDR Staining

Two hundred fifty microliters of the nuclear suspension was incubated with anti-VDR antibody (MA1-710, rat monoclonal, IgG2b; Affinity Bioreagents, Golden, CO) at 1:150 dilutions for 18 h at 4°C. The positive control used was rat IgG (catalog no. I-8015, Sigma) at a protein concentration similar to that of the antibody. After incubation, the pellet was washed with PBS and stained with 110 µl of FITC-conjugated goat anti-rat IgG (catalog no. F-6258, Sigma) at 1:80 dilutions in PBS for 35 min in the dark. Samples were counterstained with the propidium iodide solution as described above.

Flow Cytometry

Samples were analyzed in a Coulter XL flow cytometer with the standard argon ion laser excitation and filter configuration for FITC/propidium iodide dye combination. A minimum of 10,000 cells was used to generate a list-mode data file. ModFit LT 1.01 software (Verity Software House, Topsham, ME) was used for determination of the DNA index (DI) of the enzyme digested nuclei from

the tumors. The percentage of receptor-positive nuclei in the total and gated subpopulations was determined by Overton's method using EXPO-32 software obtained from Beckman Coulter (Miami, FL). As described by Overton(18), the cumulative subtraction method subtracts cells in each channel of the isotype control from cells in the corresponding channels in the test histogram. The negative differences are not replaced with a zero value, but negative values are added to positive differences in lower channels. The negative differences of higher fluorescence intensity and the positive differences of the lower fluorescence intensity cancel each other out. When cumulative subtraction is completed for all channels, a positive-difference histogram is generated by assigning a value of zero to any channel with a residual negative difference. The percentage that is positive is then calculated from the sum of the differences for all channels.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 3-µm sections were used for immunostaining. The sections were hydrated in decreasing ethanol solutions. Endogenous peroxidase was blocked with 6% hydrogen peroxidase for 3 min. The slides were rinsed in water, placed in a dish containing a target retrieval solution (DakoCytomation, Carpinteria, CA) that was heated previously to 90°C, and then placed in a steamer for 20 min to achieve optimal antigen retrieval. After a 30-min cooling period, the slides were incubated with the avidin solution and then with a biotin solution by using the Biotin Blocking System (DakoCytomation) before application of the primary antibody. ER monoclonal antibody (M 7047, DakoCytomation) at dilution of 1:25 and AR monoclonal antibody (MU256-UC, Biogenex, San Ramon, CA) at dilution of 1:20 were used for 30 min. The slides were then rinsed in buffer and incubated for 25 min with the linking solution (DakoCytomation LSAB+ Kit; biotinylated anti-mouse, anti-rabbit, and anti-goat). This was followed by a rinse in buffer and incubation with streptavidin peroxidase for 25 min. After rinsing in buffer, the slides were submerged in 3,3'-diaminobenzidine tetrahydrochloride for 5 min. One percent cupric sulfate was applied for 5 min, and the slides were counterstained with 0.1% Fast Green. The slides were then dehydrated through gradient alcohols, cleared in xylene, and coverslipped. Sections of normal female breast and normal testis were used as the positive antibody controls. The results of the immunostaining were based on the nuclear staining of tumor cells. Staining was considered positive when more than 5% of tumor cell nuclei reacted with any intensity.

RESULTS

AR Expression in Female Breast Tumors

Because previous reports indicated that AR expression may be seen even in tumors that are ER and PR negative(4), we have listed ER status (IHC) of the 23 tumors analyzed for AR expression in the present study. Of the 20 ER-positive female breast tumors, 19 had more than 20% AR-positive nuclei by flow analysis. One ER-positive tumor

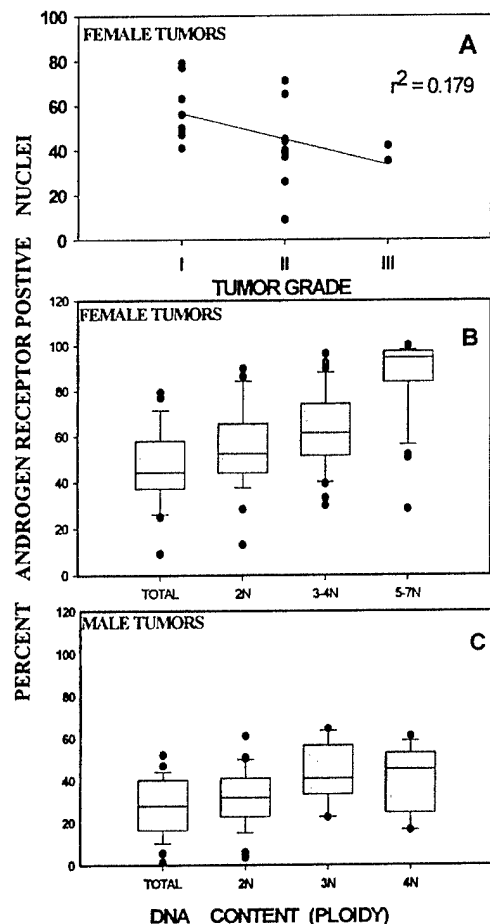


Fig. 1. Plots of the percentage of AR-positive nuclei in human female (A, B) and male (C) breast tumors. **A** correlates AR expression with tumor grade, and low-grade tumors seem to have a higher percentage of AR positive nuclei. **B** shows that diploid subpopulations in female tumors have a lower percentage of AR-positive nuclei than do aneuploid subpopulations. **C**: In male tumors AR expression is lower than in female tumors.

had fewer than 20% AR-positive nuclei. The three ER-negative tumors had 35% to 42% AR-positive nuclei.

Figure 1A correlates tumor grade with the percentage of AR-positive nuclei seen in the 23 tumors. The lower-grade tumors had a higher percentage of AR-positive nuclei than did the higher-grade tumors.

Figure 1B plots data from gated analysis of diploid and aneuploid subpopulations of the 23 tumors from female patients. The percentage of AR-positive nuclei in aneuploid subpopulations was in general higher than that of the diploid subpopulations. The mean percentage of AR-positive nuclei in diploid tumors was 55 ± 19 . In triploid subpopulations, the range was 33% to 96%, with a mean of $63 \pm 17\%$.

Figure 2 shows representative DNA histograms of tumors with diploid (Fig. 2A), near-triploid (Fig. 2B, DI = 1.6), and near-tetraploid (Fig. 2C, DI = 1.9) subpopulations. The overlay histograms (Fig. 2D-F) are of the isotype and the AR antibody-treated samples.

The histograms in Figure 2G-I show analysis of the isotype and the AR antibody-treated samples by Overton's method for determination of percentage of AR-positive nuclei. Electronic gates were used to determine the percentage of AR-positive nuclei in the subpopulations with diploid and aneuploid DNA contents. In general, Overton's analysis showed that the aneuploid subpopulations had a higher percentage of AR-positive nuclei than did the diploid subpopulations.

In addition to determining the percentage of AR-positive nuclei in a subpopulation, we compared the ratio of the mean log fluorescence channel (MFC) value of the isotype with the antibody-treated samples as a measure of AR density. In diploid tumors, the MFC ratio varied from 1.87 to 6.5. In tumors with near-triploid subpopulations, the MFC ratio was 2.28 to 6.80. In the near-tetraploid tumors, the ratio was 2.3 to 6.79.

AR Expression in Male Breast Tumors

Figure 1C shows a plot of DNA content versus percentage of AR-positive nuclei seen in subpopulations of the 33 breast tumors from male patients. In diploid tumors, the percentage of AR-positive nuclei varied from 5 to 61, with mean of 31 ± 14 . In near-triploid subpopulations, the range was 23 to 64, with a mean of 44 ± 15 . In subpopulations with near-tetraploid DNA content, the range was 16 to 61, with a mean value of 40 ± 16 .

As shown in Figure 3, female breast tumors generally had more AR-positive nuclei than did male breast tumors.

VDR Expression

Of the 17 breast tumor from female patients analyzed for VDR expression, seven had diploid DNA content. Ten of the tumors had diploid cells and distinct aneuploid subpopulations.

Figure 4 shows a plot of DNA content versus percentage of VDR-positive nuclei seen in subpopulations of the 17 female breast tumors examined. In diploid tumors, the percentage of VDR-positive nuclei varied 28 to 86, with mean of 66 ± 21 . In near-triploid tumors, the range was 68 to 91, with a mean of 70 ± 16 . In subpopulations with near-tetraploid DNA content, the range was 36 to 91, with a mean of 63 ± 20 .

DISCUSSION

Growth of normal and malignant human breast tissue is modulated by a variety of steroid hormones. Because several studies have shown that hormone receptor expression of tumor cells can have diagnostic and prognostic values, a variety of methods has been used for determination of receptor expression in breast tumors. Ligand-binding assays have been replaced by IHC studies based on the use of antibodies to the nuclear hormone receptors. In contrast, flow cytometric analysis of receptor expression in nuclei isolated from archival breast tumors offers the advantage that one can quantitatively determine the percentage of cells with receptor-positive expression and determine their antigen density by comparing the MFC of the isotype with the antibody-stained samples. The major

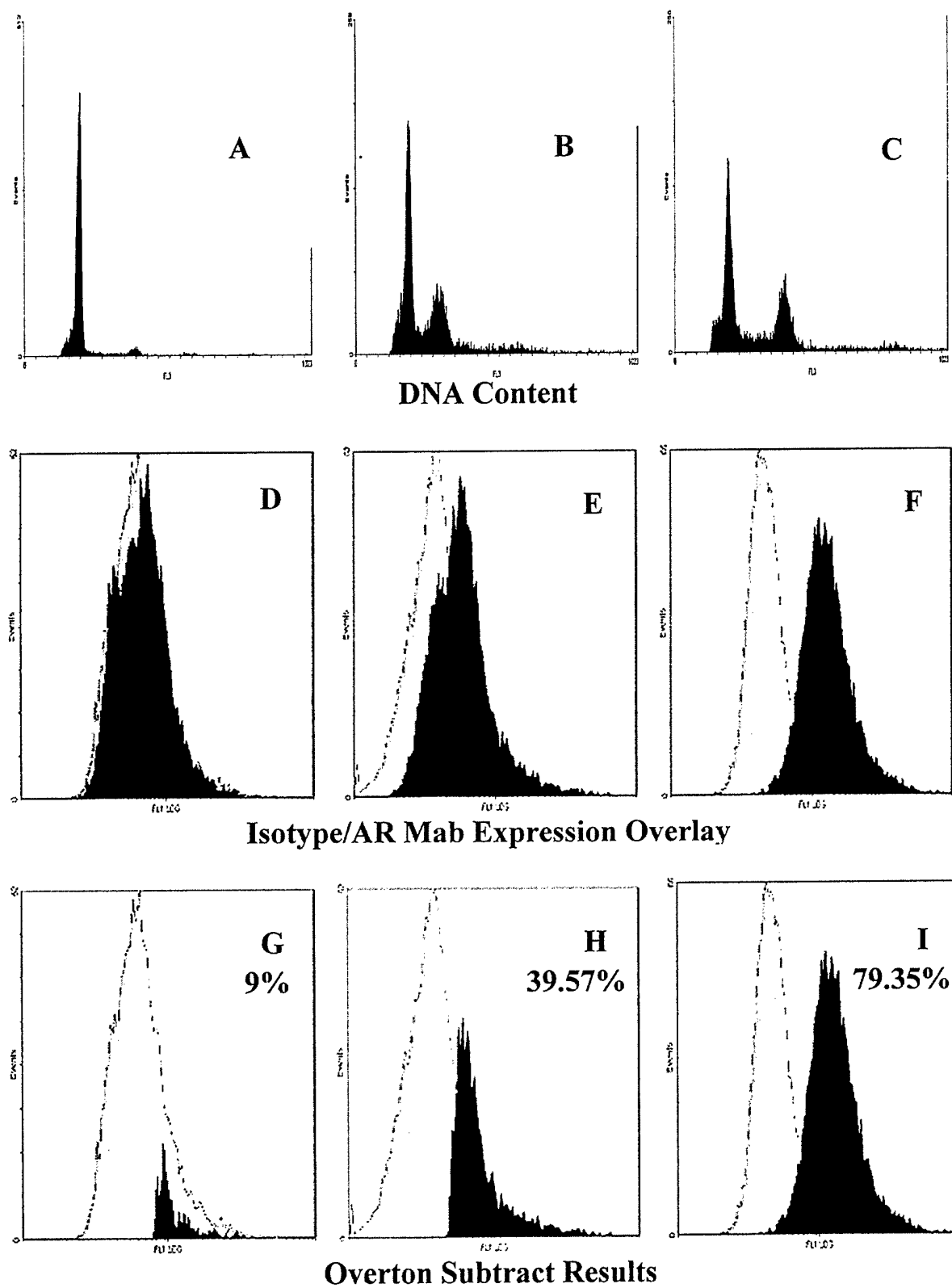


FIG. 2. AR expression in female breast tumors. **A-C:** DNA histograms of diploid and aneuploid tumors. **D-F:** Histogram overlays of the isotype and the AR antibody-treated samples. **G-I:** Histogram analysis of the isotype and antibody-treated samples by Overton's method for determination of percentage of AR-positive nuclei.

shortcoming of the flow cytometric method is that, unlike IHC, one cannot visualize the individual cells and differentiate between receptor expressions of normal and tumor cells. However, by using DNA content as a second parameter, one can differentiate between normal cells with diploid content and tumor cells with aneuploid DNA content. In tumor cells with diploid DNA content (which cannot be distinguished from normal cells on the basis of their DNA content), cellular expression of specific markers such as cytokeratin(19) or differences in the nuclear volume(20) may be used to identify tumor cells.

Most of the published work on hormone receptor expression in human breast tumors has focused on ER and PR expression. In contrast to the female hormone receptors, there is a limited number of studies on the expression of AR and VDR in human breast tumors. In general, most of the reports have indicated that majority of the human breast tumors have highly variable AR expression(21), and tumors that are ER or PR negative often show AR expression. In a recent publication, Brys et al.(22) reported that 66% and 51% of breast tumors were AR positive by reverse transcription polymerase chain reaction and western blot, respectively. The number of AR-positive cells and the AR mRNA content were higher in tumors than in normal tissues. In this study, 18% of primary breast tumors negative for ER or PR had positive AR expression. Kuenen-Boumeester et al.(4) reported that loss of ER, PR, and AR in grade III tumors was accompanied by a high proliferation index. In the present study, three ER-negative female breast tumors (by IHC) had positive AR expression by flow cytometry. At the 20% cutoff, only one of 23 tumors was negative for AR expression by flow analysis.

Human male breast tumors are relatively rare (<1%) and differ in their biological behavior from tumors of the female breast(23,24). Several reports on flow cytometric analysis of DNA ploidy in archival male breast tumors have been published. Hatchek et al.(25) reported that, of 85

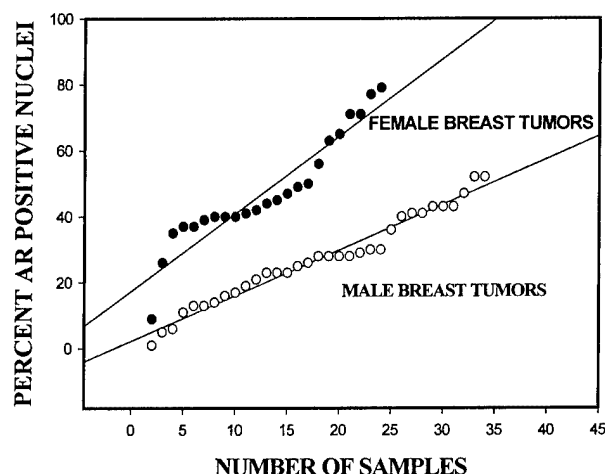


Fig. 3. Comparison of the percentage of AR-positive nuclei in female and male breast tumors.

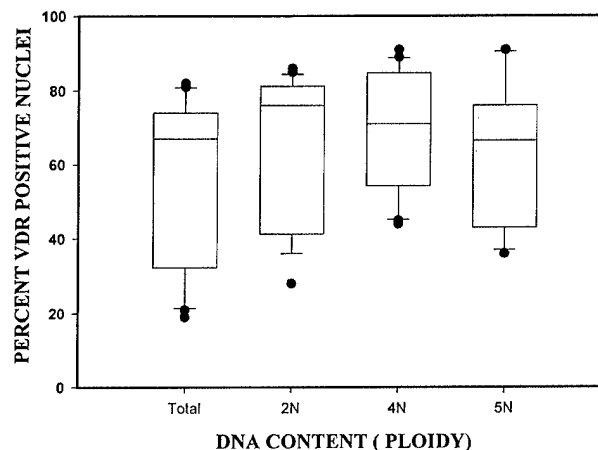


Fig. 4. Correlation of DNA aneuploidy and VDR content of subpopulations in female breast tumors.

male breast tumors examined, 57.6% were aneuploid or had multiple stem lines. Eight of 22 male breast tumors examined by Wolman et al.(26) were aneuploid. Of the six male breast tumors examined, Galasso et al.(27) found that 66% were aneuploid. In the present study, seven of 33 tumors had a DI greater than 1.0.

Several workers have reported on hormone receptor expression in human male breast tumors. Of the six tumors examined, Galasso et al.(27) found that all were ER positive. Wick et al.(28) reported that the 10 male tumors they examined had more common hormone receptor positivity at high tumor stages than did the 75 stage-matched female tumors. Rudas et al.(29) reported that the two male breast tumors they examined had strong ER, weak PR, and no AR expression. Pacheco et al.(30) reported that 75% of the male breast tumors in their study were AR positive. Pich et al.(31) found that, of the 47 male breast tumors examined, 34% were AR positive and that well-differentiated tumors were AR positive more often than the poorly differentiated tumors. Munoz de Toro et al.(32) reported that 38.5% of male breast tumors in their study were AR positive and that tumors from younger patients showed a significant lack of AR expression. They suggested that decreased androgen action within the breast tissue may contribute to earlier development of male breast cancer; moreover, once the tumor is developed, the presence of AR may contribute to tumor progression as indicated by the high proliferative activity in the AR-positive cases.

In the present study, we found AR-positive expression in 23 of 33 male breast tumors examined by flow analysis. However, AR expression in male breast tumors in general was lower than that of the female breast tumors as indicated by the percent of AR-positive nuclei and the antigen density (MFC).

Our flow cytometric studies confirmed previous IHC studies suggesting that most breast tumor cells have positive VDR expression(5,6). In contrast to AR, VDR expression did not seem to increase in aneuploid female breast

tumors, and diploid subpopulations had more VDR-positive nuclei than did multiploid subpopulations.

In general, flow cytometric data in the present study on AR expression in male and female breast tumors do not correspond well with labeling of these tumors as AR positive or negative by IHC. In contrast to IHC studies in which an observer can use 5% as the cutoff for calling a tumor receptor positive or negative, in flow analysis, the range of nuclei with positive expression (after subtraction of the isotype values) can be extensive. In a previous study(16), we used AR-negative (PC-3) and AR-positive (LnCap) cell lines to determine the cutoff values that could be used to identify receptor-positive and -negative tumors. In AR-negative PC-3 cells from log and plateau phase cultures, the percentage of AR-positive nuclei was less than 20, and the ratio of MFC was less than 2. In contrast, in the AR-positive LnCap cells, the percentage of AR-positive nuclei was greater than 30 and increased with increased confluence, reaching a maximum of 94% positive nuclei and a MFC value of 11 in plateau phase cultures. Thus, one could use these values to differentiate between receptor-positive and -negative tumors by flow cytometry. On this basis, 23 of 33 male breast tumors analyzed in the present study (Table 2) were AR positive by flow cytometry, whereas IHC identified only six of 33 tumors as AR positive.

One of the reasons for discrepancy between the IHC and flow cytometric analysis could be that, whereas the IHC data are based on enumeration of tumor nuclei alone, the flow analysis measures antigen reactivity of the tumor and the normal epithelial nuclei. Thus, gated analysis (using DNA content or nuclear volume) and use of specific markers that can differentiate normal from tumor cells could be used to refine the flow cytometric method for analysis of nuclear hormone receptors.

As shown in the present study, flow cytometric analysis of nuclear hormone receptor expression can be routinely performed in archival formalin-fixed, paraffin-embedded tumors. Data obtained from these studies can be used for correlative studies comparing expression of different cellular markers and possibly for retrospective studies seeking to correlate receptor expression with clinical outcome and response to therapy.

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Methodological Report

Flow Cytometric Analysis of Electronic Nuclear Volume and DNA Content in Normal Mouse Tissues

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KEY WORDS

electronic volume, coulter volume, DNA content, cell cycle, DNA flow cytometry

ABBREVIATIONS

TM	trademark
EV	electronic volume
hrs	hours
min	minutes
FBS	fetal bovine serum
PBS	phosphate buffered saline
PI	propidium iodide
nm	nanometers
TRBC	trout red blood cells

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ABSTRACT

Light scatter is used in flow cytometry for identification of cells based on their size and/or granularity. However, forward light scatter is not an accurate measure of cell size. The measurement of Electronic Volume (EV) by Coulter principle is more accurate. However, EV cannot be measured on most of the commercially available flow cytometers. We have described the development and applications of a flow cytometer that can simultaneously measure Electronic Nuclear Volume (ENV) and DNA content. In the present study we have used a commercially available NPE QuantaTM for measuring EV and DNA content of different normal mice tissues.

Fresh/frozen or formalin fixed-paraffin embedded tissues from mice were processed for isolation of nuclei, which were then analyzed for EV versus DNA content. By using these two parameters, distinct sub-populations were identified in liver, thymus, small intestine and bone marrow.

Dual parametric analysis of EV versus DNA content can be a valuable technique for identification of sub-populations in heterogeneous cell mixtures such as those of complex tissues like bone marrow, intestine and tumors. The methods established are rapid and can provide valuable data for identification and characterization of sub-populations for cell cycle analysis by flow cytometry.

INTRODUCTION

Light scatter is used in laser flow cytometry for identification of cell populations based on their approximate size and granularity. Forward versus side scatter has been a useful parameter particularly for gating of lymphocytes in phenotypic analysis. Earlier reports have shown that in spite of its utility and wide spread use, light scatter is not an accurate measure of cell volume or diameter.^{1,2} In contrast, measurement of Electronic Volume (EV) by the Coulter principle is a much more reliable and accurate method for determination of cell volume.³ However, for a variety of reasons, Coulter volume measurement is not offered on the currently available commercial flow cytometers and cell sorters.

In earlier reports, we have described the development and application of a flow cytometer that can simultaneously measure nuclear DNA content and EV.⁴⁻⁶ We have shown that correlated measurement of EV and DNA content can identify sub-populations which can not be detected by analysis of light scatter versus DNA content alone.⁶ In the present report, we describe the use of EV in conjunction with DNA content for analysis of fresh and formalin fixed-paraffin embedded normal mice tissues.

MATERIALS AND METHODS

Eight-week-old male C57BL/6 mice were purchased from commercial sources and housed in the Animal Care facility at the University of Miami School of Medicine. Mice were anesthetized by exposure to CO₂ and sacrificed for collecting the following tissues: Spleen, bone marrow, liver, intestine, thymus, lung and pancreas. Tissues were stored frozen in a low temperature freezer (-80°C) or processed after fixation in formaldehyde and embedding in paraffin.

Fresh or Frozen Tissue. Small pieces of the dissected tissue were placed in a 50 mm Petri dishes with 2 mL of Nuclear Isolation Media (NIM)- DAPI staining solution [NIM-DAPI, NPE Systems, Pembroke Pines, FL]^{7,8} and minced with scalpel. After light pipetting, the minced tissue was filtered thru 25 µm Filter Tip (NPE Systems) and trout red blood cells (TRBC), (DNA Reference Calibrator, NPE Systems) were added as an internal standard. The final nuclear concentration was adjusted to approximately 1–2 x 10⁶ cells/mL. Samples were analyzed after 10–30 min of staining on ice.

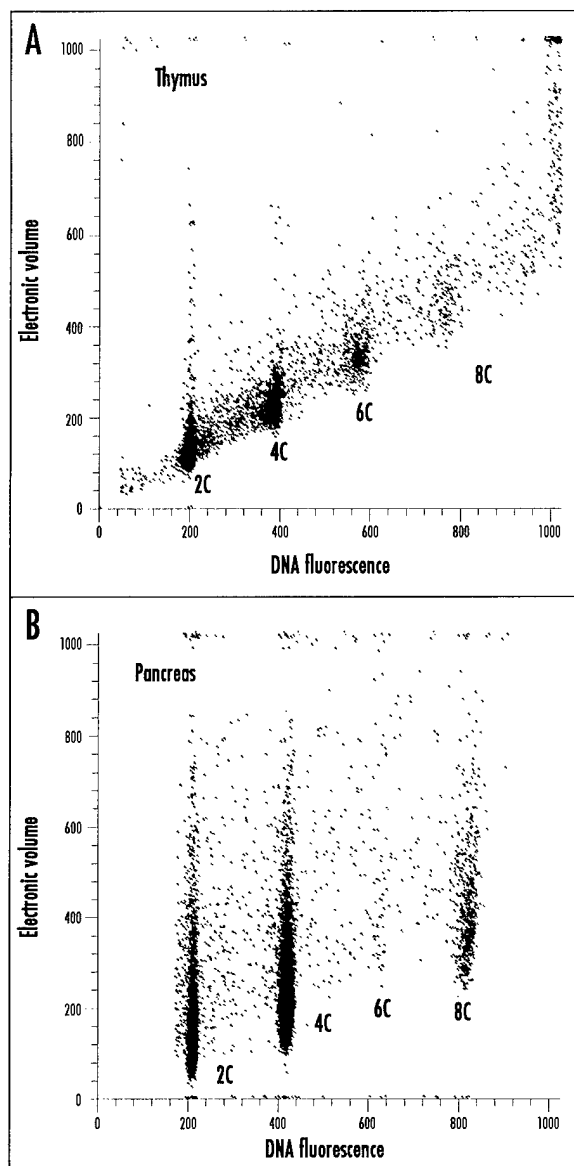


Figure 1. EV versus DNA content plots of nuclei from fresh thymus (A), and pancreas (B). In thymus and pancreas, nuclei with 2-8 C DNA content were seen. TRBC were not included as internal standard in these samples.

Paraffin Embedded Tissues. Samples were fixed in 10 percent buffered formaldehyde solution for 24 hrs and washed before dehydration thru an ascending alcohol series. Following clearing in xylene, tissues were embedded in paraffin. Sections (50 μ m) were cut and processed for deparaffinization in xylene or Citri-Solv (Fisher Scientific, Pittsburg, PA). After washing, sections were rehydrated and washed with distilled water for 10 min to overnight at room temperature.

Nuclear Isolation and Staining. Nuclei were isolated by digestion in a propriety enzyme cocktail (NPE PARA-KIT, NPE Systems) or in pepsin as described earlier at 37°C for 30 min.⁹ Samples were gently vortexed every 10 min and the proteolytic action was stopped by the addition of an equal volume of 3% fetal bovine serum (FBS) in phosphate buffered saline (PBS). After centrifugation, the nuclear pellets were resuspended in NIM-DAPI as described above. Preparations were examined under a phase contrast microscope to check on integrity of the isolated nuclei.

Minced (fresh or frozen) tissues and deparaffinized samples were also stained with propidium iodide/hypotonic citrate¹⁰ for analysis in a Coulter XL-MCL cytometer (Beckman Coulter, Inc. Miami, FL).

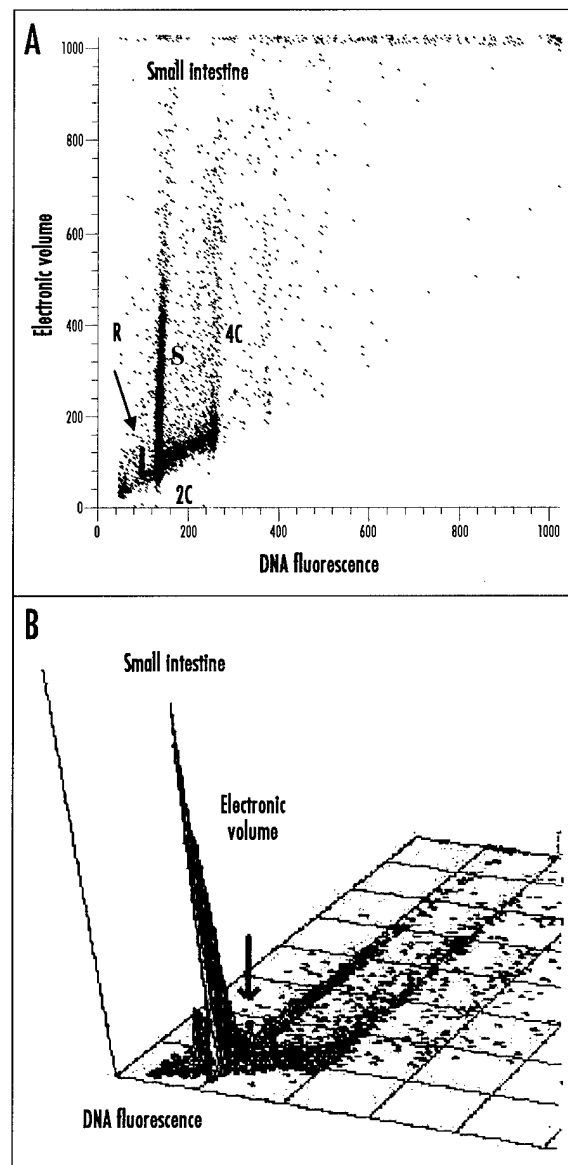


Figure 2. A dot plot (A) and an isocontour plot (B) of a fresh sample of small intestine. TRBC (R) were used as internal DNA standard. In small intestine, most of the nuclei with S and G₂/M DNA content (arrow) belonged to the population with small EV.

Flow Analysis. Propidium iodide (PI) stained samples were analyzed by collecting forward scatter versus DNA propidium iodide fluorescence. A minimum of 10,000 cells was analyzed to collect list mode data. NIM-DAPI stained samples were run on a NPE QuantaTM flow analyzer fitted with a mercury arc lamp (HBO), UG1 filter (for 365 nm excitation), a 410 nm dichroic mirror and a 450/55 nm emission filter. EV and DAPI-DNA fluorescence of a minimum of 10,000 nuclei was collected in list mode files.

Data Analysis. WinList 3D (Verity Software House, Topsham, ME) and WinMidi vs 2.8 software (downloaded from <http://facs.scripps.edu/>) were used for graphics. The ModFit LT software (Verity Software House) was used for cell cycle analysis.

RESULTS

Fresh Tissues. Figure 1A and 1B shows dot plots of EV versus DNA content of nuclei isolated from mouse thymus and fresh pancreas and stained with DAPI. Populations with 2-8 C DNA content were seen in these plots. It is remarkable to note differences in EV of the nuclei from the

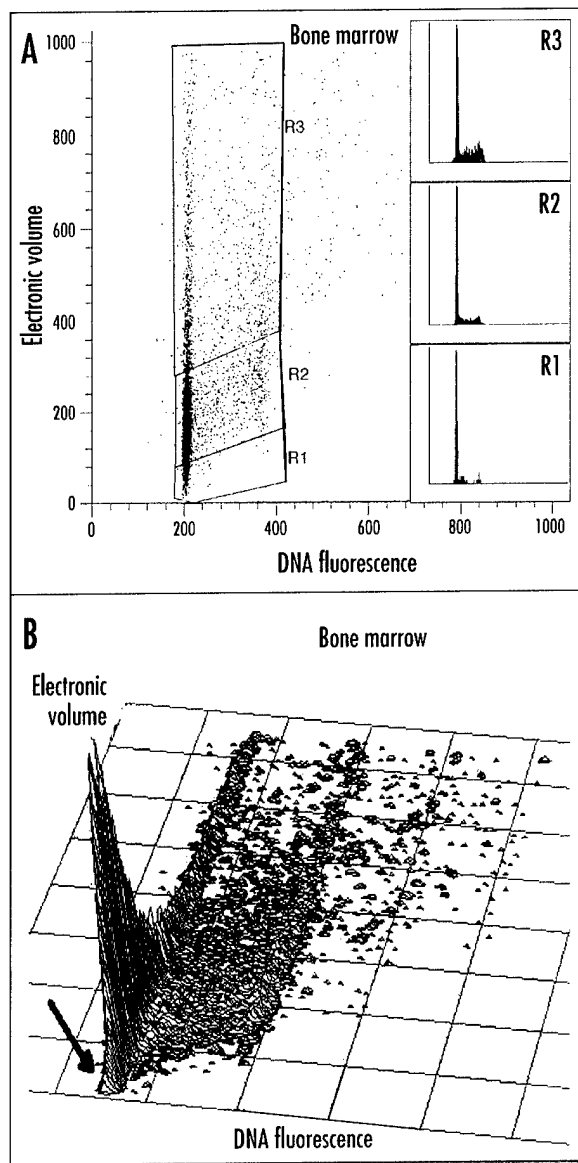


Figure 3. Dot plots and DNA histograms of gated sub-populations in a bone marrow sample (A) and an isocontour plot of the same (B). The histograms insets in A belong to R1, R2 and R3 from the dot plot in A. The arrow in B shows that most of R1 nuclei are in G_0/G_1 , while most of the nuclei with S and G_2/M DNA content were in R2 and R3 gate of the dot plot (A and B).

homogeneous thymus with 2C to 8C DNA content and the nuclei with 2C, 4C and 8C DNA content in the functional heterogeneous pancreas. In pancreas, (Fig. 1B) the nuclei with the diploid (2C) DNA content were approximately 40 % of the total nuclear population. The presence of nuclei with 2C and 4C DNA content was confirmed in samples stained with propidium iodide/hypotonic citrate and analyzed in a Coulter XL Cytometer (data not shown) as well as by microscopic examination where distinct populations of small, medium and large nuclei were seen.

In contrast to the small EV of the nuclei from the thymus (Fig. 1A), nuclei isolated from the small intestine had a broad EV distribution as shown in (Fig. 2A). As seen in the dot plot 2A and isocontour plot 2B (arrow), most of the proliferating nuclei with S and G_2 DNA content had small EV. These plots show the importance of the EV parameter for characterization of the different sub-populations in a heterogeneous tissue.

In dot plot 3A and isocontour plot 3B, nuclei from fresh bone marrow are shown. On the basis of EV, we can recognize three distinct populations:

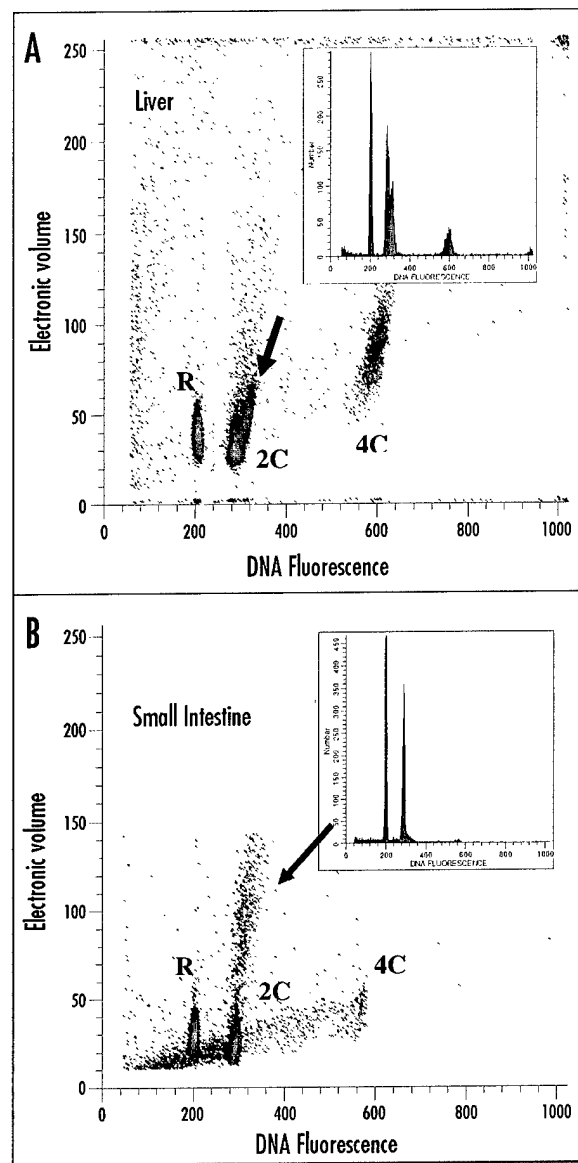


Figure 4. EV versus DNA content plots of formalin fixed/paraffin embedded liver (A) and small intestine (B). TRBC (R) were added to the samples as internal standard. In liver, two distinct nuclear sub-populations with G_0/G_1 DNA content (A, arrows) were seen. In small intestine, most of the proliferating nuclei had small EV (B). A sub-population of nuclei with large nuclear volume (B, arrow) had mostly nonproliferating cells.

1) a small sub-population of nuclei with diploid (2C) DNA content (R1 region which constitute 5.8% of the total NUCLEI) and do not have a corresponding population of nuclei with S and G_2 DNA content. 99.9% of these nuclei (arrow in Fig. 3B) are in G_0/G_1 of the DNA cell cycle. In contrast, nuclei in the R2 region with medium EV had 24% of the nuclei with S-phase DNA content (Fig. 3A-R2). The nuclei with larger EV from the R3 region constituted 28.9% of the total nuclei population and had 36.32% nuclei with S-phase DNA content (Table 1).

Formalin Fixed/Paraffin Embedded Tissues. Histograms of the formalin fixed/paraffin embedded tissues (after enzyme digestion of the 50 μ m sections) were generally similar in appearance to those obtained from the fresh/frozen tissues.

Figures 4A and B are dot plots of EV vs. DNA content of formalin fixed/paraffin embedded liver and small intestine mixed with TRBC (R). Insets show corresponding DNA histograms. Comparison of the EV vs. DNA in liver nuclei (Fig. 4A arrow) shows the presence of two distinct

Table 1 DATA OF CELL CYCLE ANALYSIS OF FRESH BONE MARROW*

	R1	R2	R3
Percentage of total nuclei (%)	5.8	65.3	28.9
G ₀ /G ₁ (%)	99.9	70.80	56.98
S (%)	—	24.01	36.32
G ₂ (%)	—	5.19	6.71

*The histograms of each region: R1, R2, and R3 are shown in Figure 3A.

nuclear populations with G₀/G₁ DNA content. EV of the TRBC and the liver diploid nuclei was similar.

In small intestine (Fig. 4B), distinct sub-populations could be identified on the basis of differences in EV and DNA content. The diploid nuclei with corresponding nuclei with S and G₂/M DNA content had EV similar to that of the TRBC (R). However, an arrow indicates presence of diploid nuclei with large EV, which do not have a corresponding population of cells with S and G₂ DNA content.

DISCUSSION

Data in the present report shows that measurement of EV in fresh/frozen or paraffin embedded archival tissues can provide important information on sub-populations in a heterogeneous population which can not be obtained by monitoring of light scatter alone. By incorporation of TRBC as an internal standard in each sample, we have checked and compared both the EV as well as DNA content of the normal tissues. Linearity of measurement was seen for both DNA and EV measurements and fixation in formalin did not affect monitoring of EV. Data collected from analysis of the different normal tissues was in line with what is known about presence of different sub-populations in these tissues. For example in thymus, we found several populations with multiploid DNA content. As our nuclear isolation and staining solution contains a detergent (NP-40), we believe the multiploid peaks are not caused by clumping of nuclei. Similarly, in the pancreas both staining with propidium iodide/hypotonic citrate and microscopical examination confirmed the presence of cells with multiploid DNA content. In liver one expects the nuclei of reticuloendothelial cells and lymphocytes to have smaller EV than the nuclei of the liver parenchymal cells. These nuclei are not only larger in size but also show more DAPI fluorescence than lymphocytes. In nuclei from small intestine, the proliferating sub-population seems to stand distinct from the nuclei with large EV, which presumably belong to the epithelial cells.

In parallel studies (manuscript in preparation), we have used monitoring of EV vs. DNA content to study a variety of body fluids suspected to have malignant cells. Our preliminary data shows that by combining measurement of EV with other parameters such as DNA content or phenotypic markers, one can identify sub-populations of clinical interest.¹¹

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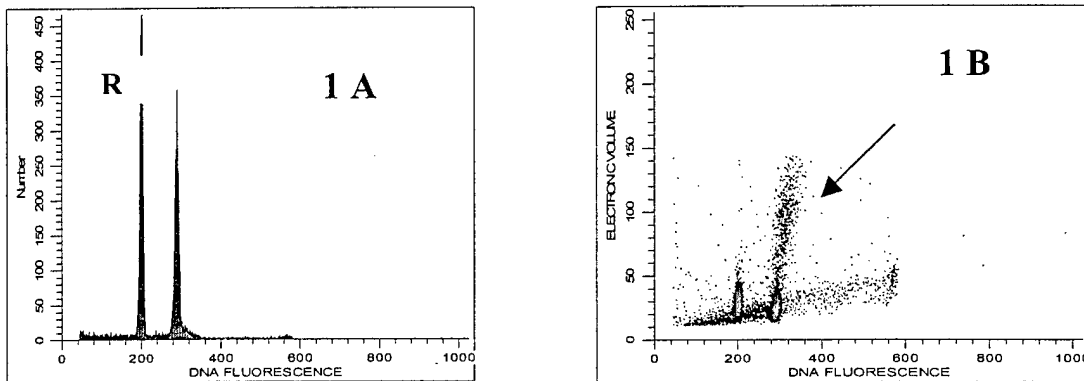


Figure 1. Figure 1A shows a high resolution DNA histogram of nuclei from small intestine and trout red blood cells (R). Figure 1B shows a DNA vs. Electronic volume plot of the same sample. The arrow points to a sub-population of large non-cycling epithelial nuclei. The smaller basal cell nuclei showed proliferating S and G₂M nuclei.

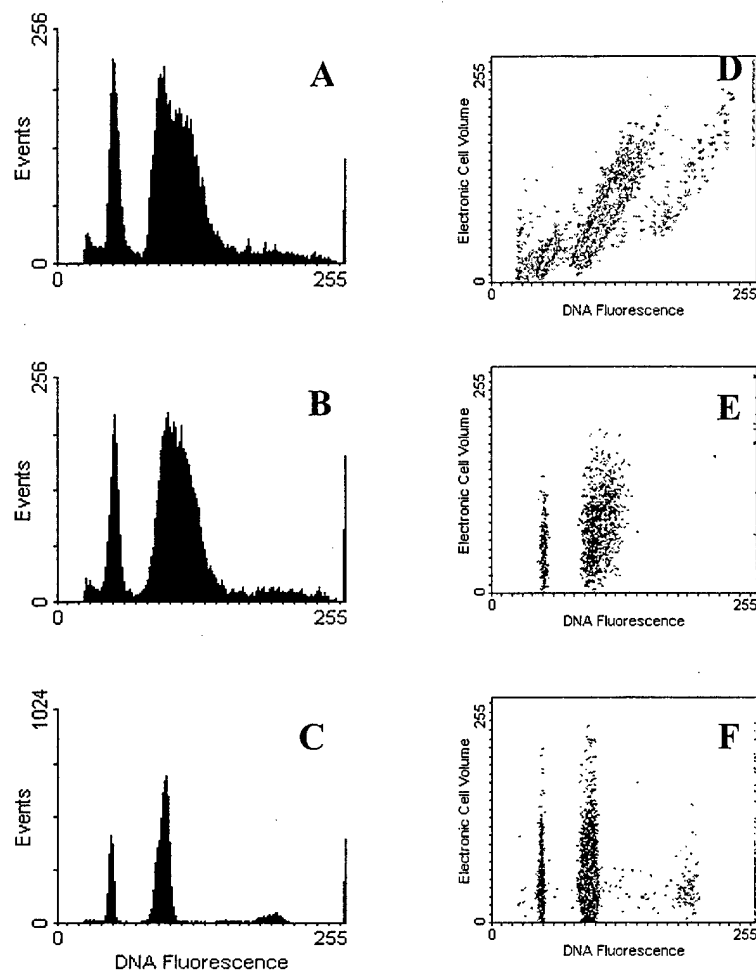


Figure 2. Figure 2 shows the effect of excess DAPI on the DNA histogram.

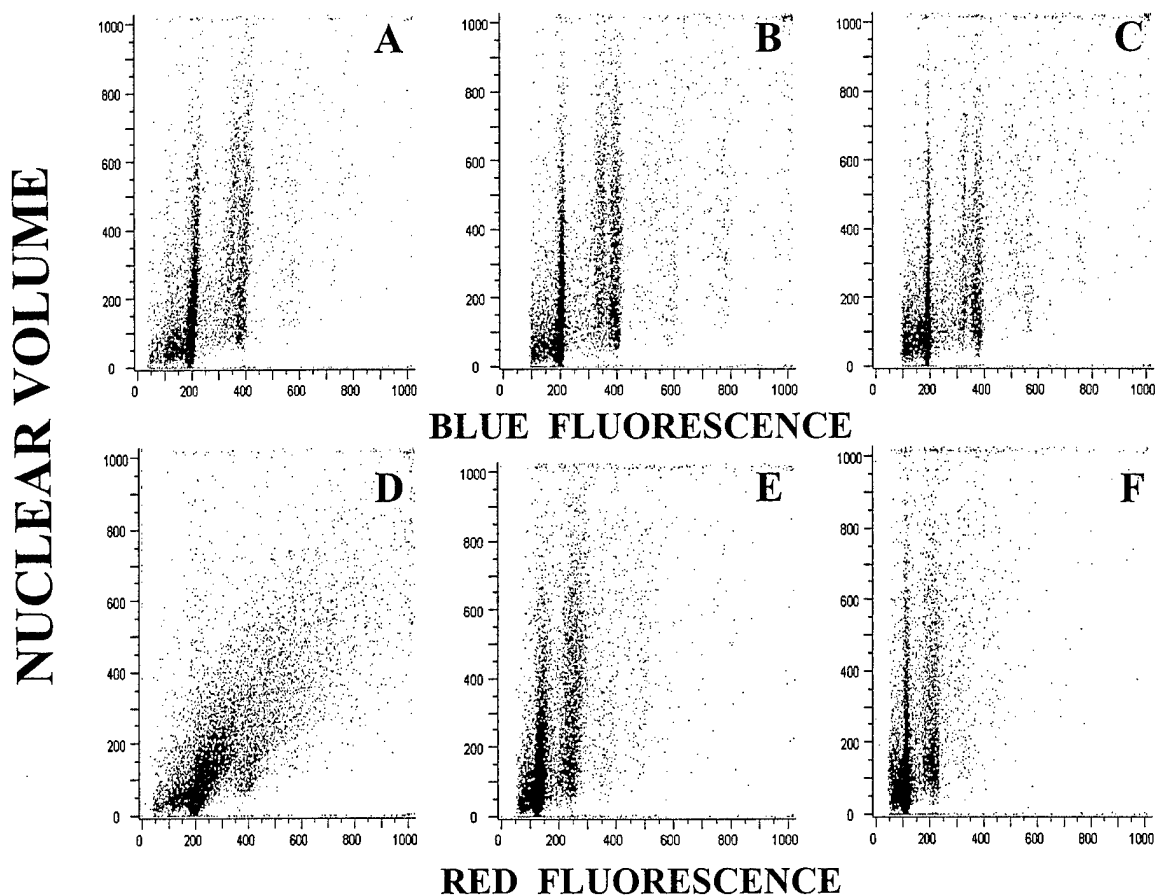


Figure 3. Figure 3 shows the Red and Blue emission of nuclei stained with NIM DAPI (A,D), a 1:2 dilution of NIM DAPI (B,E), and our DAPI (C,F).

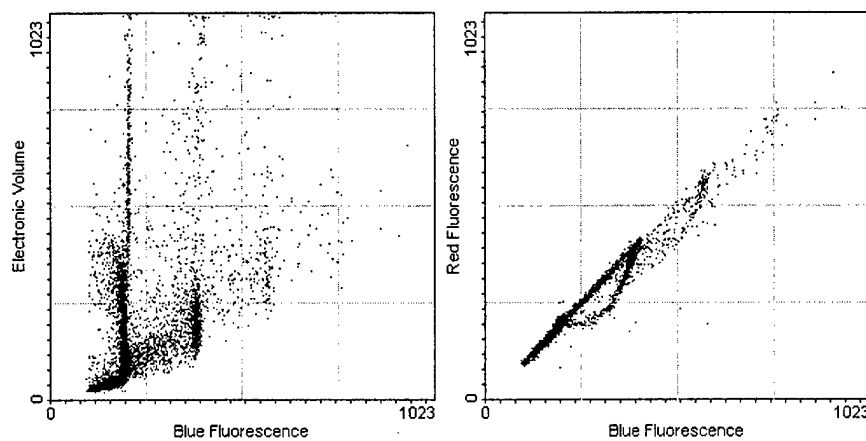


Figure 4. Figure 4 shows two distinct S-phase sub-populations based on differing Red/Blue DAPI emissions.

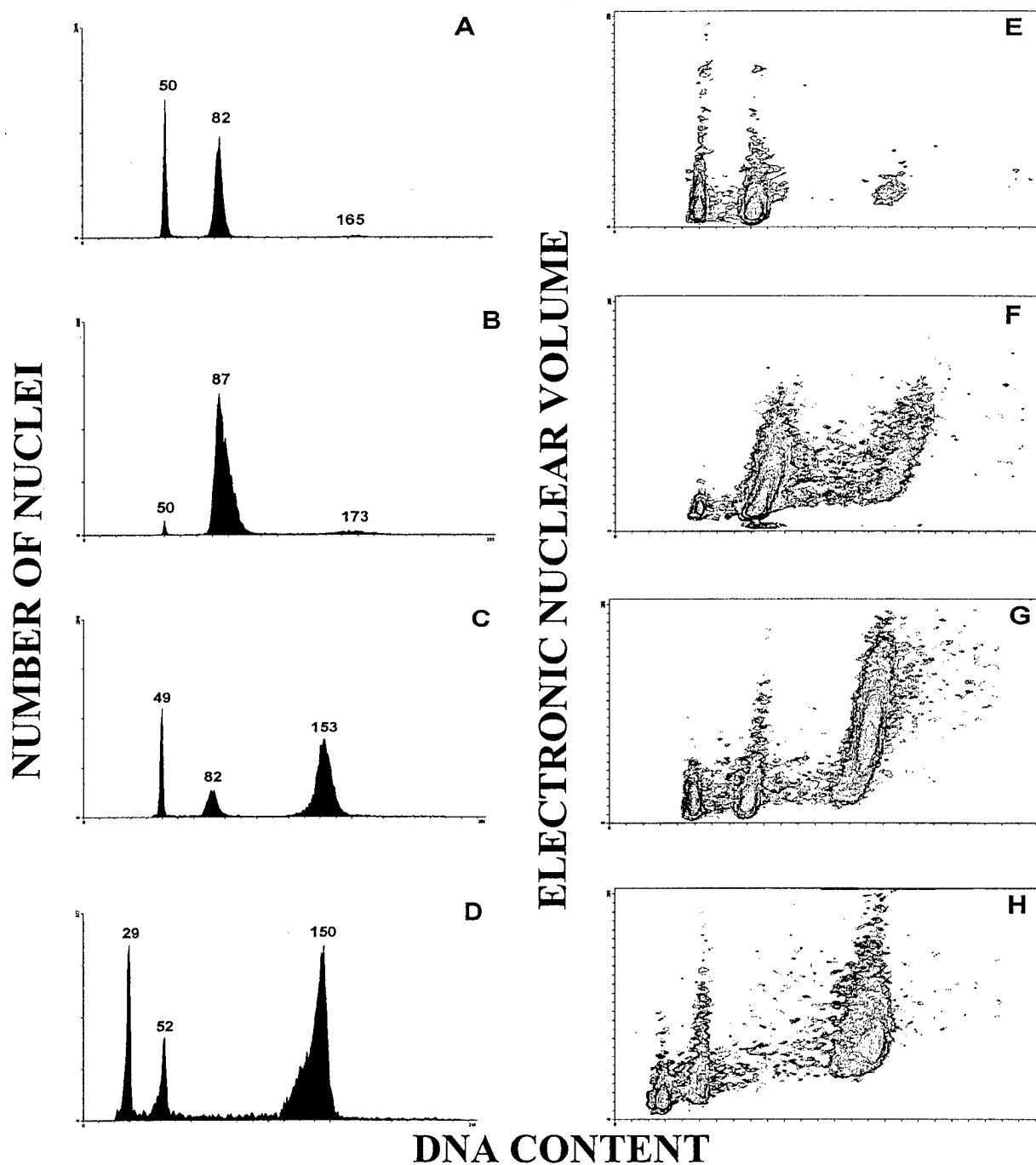


Figure 5. DNA histograms (A-D) and contour plots of DNA vs. Electronic nuclear volume (E-H) are of nuclei isolated from normal breast tissue (A, E), and from DCIS biopsies (B-D, F-H). Trout red blood cells were added as an internal standard and recorded at channel # 50 (A-C) or at channel # 29 (D). In F, ENV of the tetraploid nuclei was similar to that of the diploid nuclei. In G, the tetraploid nuclei were 2 to 4 fold larger in nuclear volume. In contrast, the aneuploid nuclei in H had relatively smaller nuclear volume.

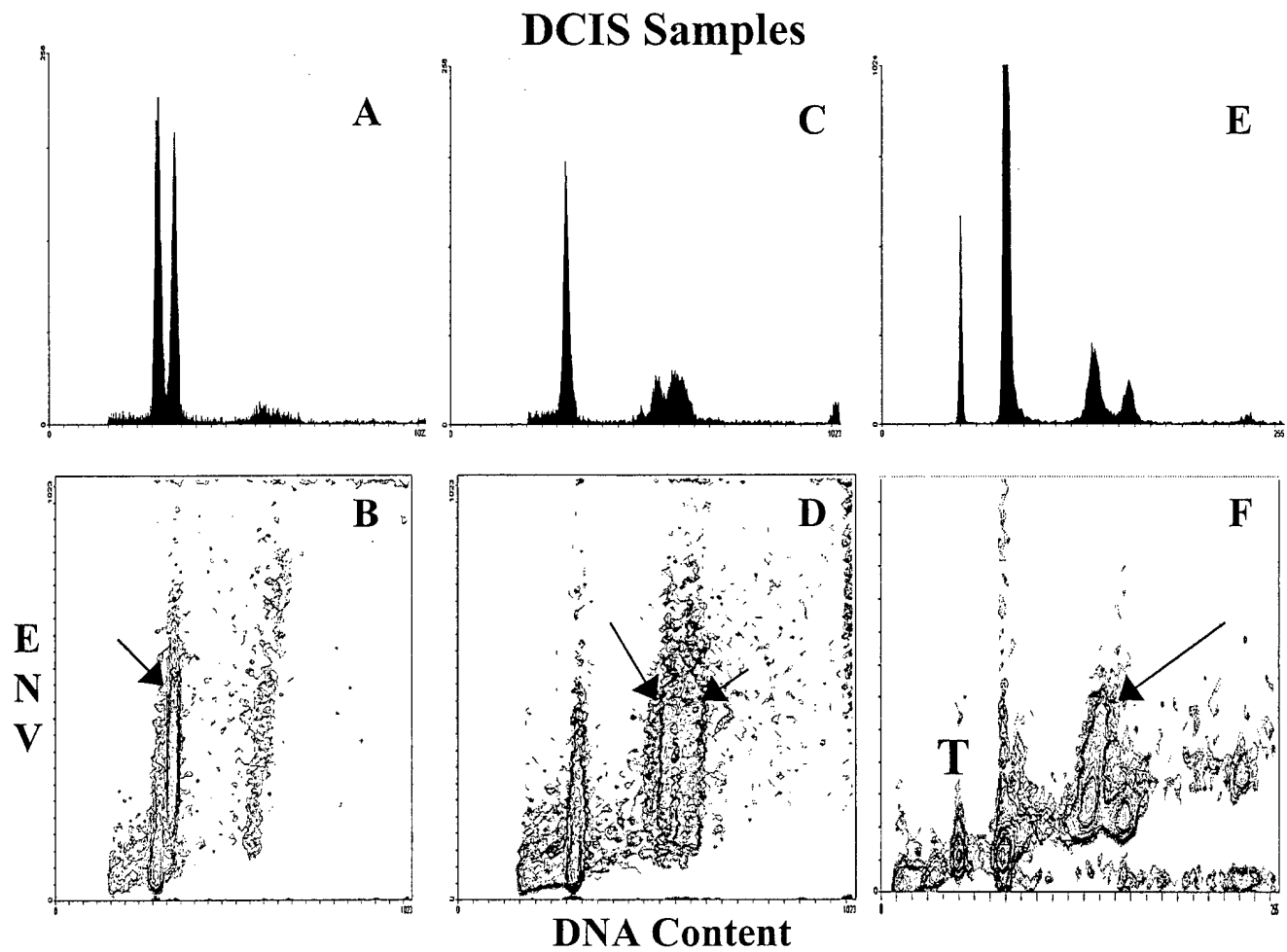


Figure 6. Figure 6 shows DNA content (A,C,E) and contour plots of nuclear volume vs. DNA content (B,D,F) of DCIS samples. Arrows point to the presence of nuclei with large volume.

PR EXPRESSION IN BREAST TUMORS
Formalin fixed/Paraffin embedded

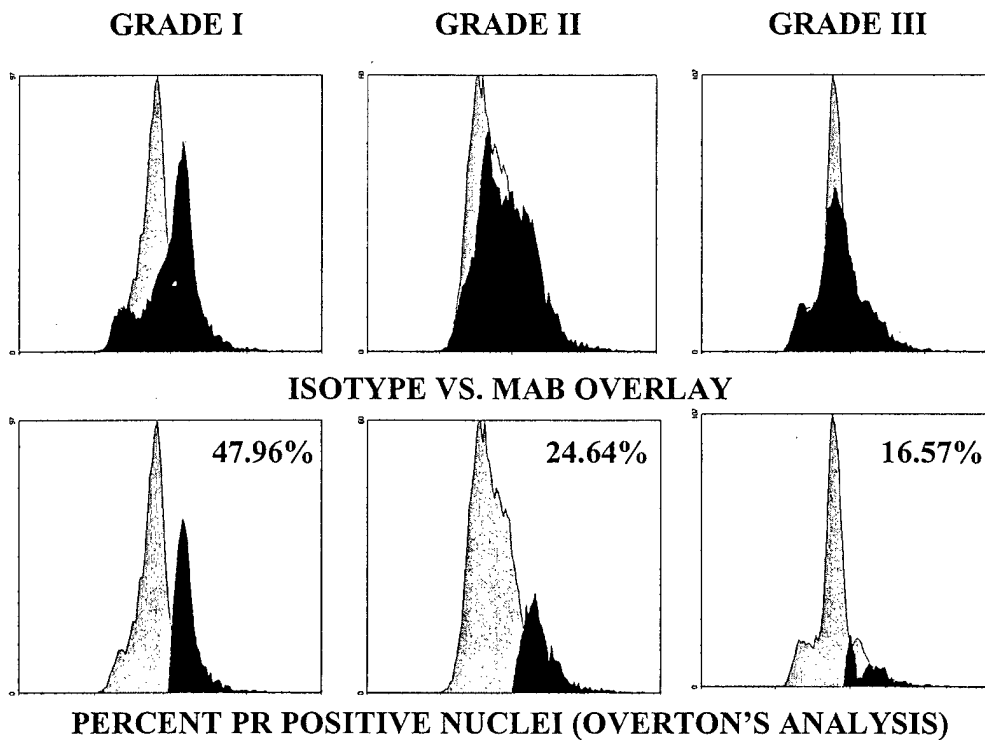


Figure 7. Figure 7 shows histogram overlay and Overton subtraction plots of PR expression in three grades of DCIS breast tumors.

Fresh/Frozen

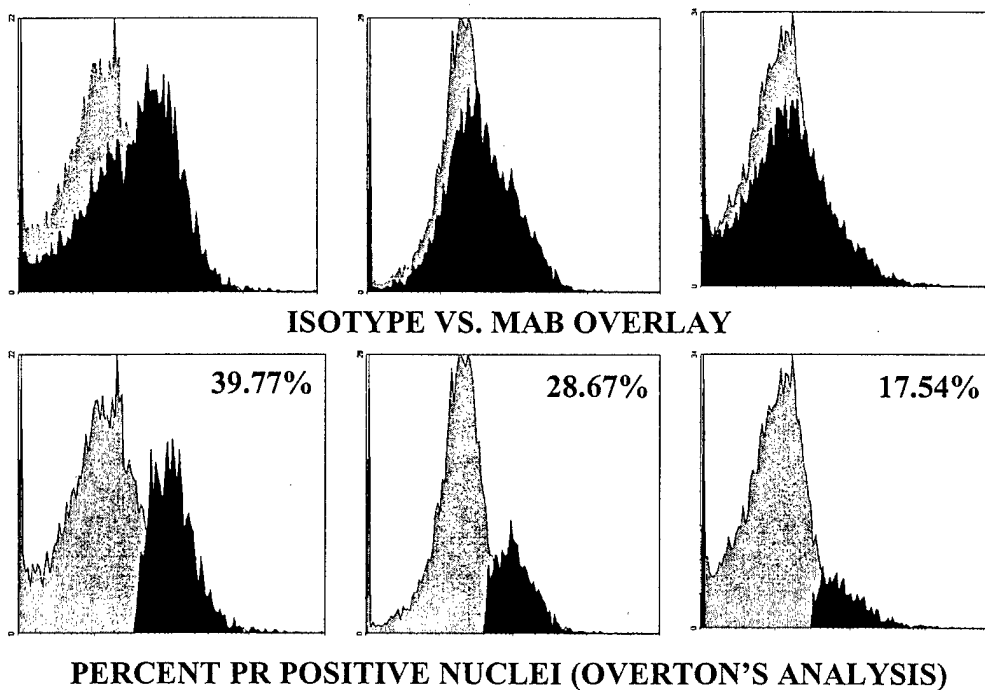


Figure 8. Figure 8 shows histogram overlay and Overton subtraction plots of PR expression in three different frozen breast samples.